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Derangements in the cell cycle machinery contribute to uncontrolled cell growth and tumorigenesis. Regulators of G1 progression including cyclin D1 and the cyclin-dependent kinase inhibitors p21 and p27 appear to be of particular importance in the pathogenesis of breast cancer. We suggest that aggressive breast cancer will involve both (i) overexpression of cyclin D1 and (ii) the failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims are designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. We have generated mouse embryo fibroblasts (MEFs) that stably express a tetracycline-regulated cyclin D1 construct. In contrast to the data in NIH-3T3 cells, there was no compensatory upregulation of p21 in MEFs despite significant overexpression of cyclin D1. This may be due to the acquisition of p53 mutations. In addition, human breast cancer tissues have been analyzed for cyclin D1, p27 and Ki67 expression. Following optimization of p21 staining and analysis, correlation with prognostic factors will be performed.

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Catherine Fullerton 11/9/99

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INTRODUCTION

Proteins that regulate progression through the cell cycle have increasingly been implicated as oncogenes. This is particularly true for those which control progression through the G1 phase, including cyclin D1 and the cyclin-dependent kinase (cdk) inhibitors $p21^{cip1}$ (p21) and $p27^{kip1}$ (p27). Cyclin D1 upregulation occurs as a result of gene amplification and/or mRNA overexpression in a substantial proportion of human breast cancers (1-5). Overexpression of cyclin D1 in transgenic mice leads to breast hyperplasia and multifocal carcinoma (6). Alternatively, in breast as well as other cancers, the cdk inhibitors appear to function as tumor suppressors. Thus, lower than normal protein levels correlate significantly with tumor aggressiveness, histologic grade, and decreased overall patient survival (7-11).

As positive and negative regulators of proliferation, respectively, the levels of cyclins and cdk inhibitors relative to each other determine whether progression through G1 phase proceeds (12-16). In addition, as key factors controlling G1 progression, all three are regulated by extracellular stimuli, including growth factors and cellular adhesion to the extracellular matrix (17,18). Such actions are responsible for the anchorage- and mitogen-dependence of G1 progression in normal cells. Conversely, anchorage- and mitogen-independence is the hallmark of tumorigenesis.

Whether the aberrant levels of cyclin D1, p21, and p27 result in altered cell cycle progression and subsequent proliferation remains to be established. Overexpression of cyclin D1 in cells can result in similar or even delayed cell cycle kinetics, compared to normal mammary epithelial cells (4, 19, 20). Moreover, the overexpression of cyclin D1 fails to induce anchorage-independent growth, the best in vitro correlate of tumorigenicity. Our preliminary data show that the consequences of cyclin D1 overexpression will be most apparent when examining anchorage-independent growth because that is the condition in which normal levels of cyclin D1 become rate-limiting. In addition, our data show that the overexpression of cyclin D1 in NIH-3T3 cells leads to a compensatory increase in the cdk inhibitor p21, and that this compensatory increase can counteract the expected cyclin D1 effect on anchorage-independent Rb phosphorylation and cell cycle progression. Thus, we proposed that the value of cyclin D1 overexpression as a diagnostic indicator for breast cancer is weakened by the compensatory upregulation of cdk inhibitors (CKIs) that can occur in breast cancer cells. We further suggest that aggressive breast cancer will involve both (i) overexpression of cyclin D1 and (ii) the failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims outlined below are designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. Aim 1 is to show that compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression in inducing anchorage-independent growth. Aim 2 is to determine if compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression on tumor formation. Aim 3 is to determine if the overexpression of cyclin D1 in breast cancer cell lines has a

more pronounced growth effect if the cells have also lost their ability to upregulate CKIs. Aim 4 is to examine the relative expression of cyclin D1 and CKIs in a series of breast cancer biopsies.

BODY

Aim 1: Test the hypothesis that the compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression in inducing anchorage-independent growth.

Task 1. Complete characterization of adhesion-dependent phenotype of wild-type, p21, and p27 null mouse embryo fibroblasts (MEFs).

As demonstrated previously in the last report, we have found that both p21-null and p27-null MEFs have partially lost the ability to down-regulate cyclin E-cdk2 activity when stimulated in suspension. However, they retain normal adhesion requirements for cyclin D1 expression, Rb phosphorylation, and cyclin A expression. These cell lines thus exhibit an adhesion-dependent phenotype despite the knock-out of one or the other cdk inhibitors.

Task 2. Obtain tetracycline-cyclin D1 transfectants in mouse embryo fibroblasts. Analyze the tetracycline-cyclin D1 cells (wild-type and knock-out) for their ability to undergo anchorage-independent growth. Compare the rates and extent of Rb phosphorylation, cyclins D1 and A expression, and p21 levels when the transfectants are cultured in the presence and absence of substratum.

At the last progress report, we indicated that MEFs were not efficient in their ability to form stable transectants. However, we have been able to transfect early passage mouse embryo fibroblasts (MEFs) with a tetracycline-repressible cyclin D1 cDNA and isolate several clones that show tetracycline-regulated expression of cyclin D1. (In this system, tetracycline represses the expression of the ectopic cDNA and removal of tetracycline causes its induction.) The general goal is to determine if the induction of cyclin D1 leads to increased expression of p21 as we observed in NIH-3T3 cells.

We have isolated three distinct clones that show tetracycline-regulated expression of cyclin D1, clones M13, M14 and M18 (Fig 1). The clones differ in the basal level of cyclin D1 expression (observed when cultured in the presence of tetracycline) and in the degree to which cyclin D1 expression is properly adhesion dependent (regulated correctly in clones M13 and M14, but apparently not so in clone M18). Importantly, all three clones show strong induction of cyclin D1 upon removal of tetracycline. p21 expression, as expected from our previous studies (17) is upregulated by culturing the cells in the absence of a substratum. However, p21 expression is not altered by the induction of cyclin D1 (Fig 1, compare p21 levels in the presence an absence of tetracycline). The inability of

cyclin D1 overexpression to alter p21 is seen in both adherent and nonadherent cells from all three clones. Given these unexpected results, the proposed follow-up experiments involving tetracycline-regulated expression of cyclin D1 in MEFs from p21 or p27 null mice are no longer justified (aim 2 in the original application).

Task 3. Analyze the mechanism by which cyclin D1 regulates the expression of p21.

There appears to be fundamental differences in the way p21 is influenced by the levels of cyclin D1 in NIH-3T3 cells compared to MEFs. We are considering the possibility that p21 is not being induced because the MEFs are acquiring p53 mutations as a consequence of time in culture. We plan to use the NIH-3T3 cell system to determine if the induction of p21 is due to changes in abundance of its mRNA. This result would support the idea that p53 mutations might be preventing us from seeing the cyclin D1 effect in long-term MEF cultures.

Aim 2: Determine if compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression on tumor formation.

As stated above, we observed no changes in the levels of p21 despite potently inducing cyclin D1 overexpression in MEFs. This lack of effect in MEFs is critical to approaching Aim 2 as this is the cell line where p21- and p27-null cells have been produced. The proposed experiments, to determine the consequences of compensatory p21 upregulation on tumor formation are therefore, no longer justified.

Aim 3: Determine if the overexpression of cyclin D1 in breast cancer cell lines has a more pronounced growth effect if the cells have also lost their ability to upregulate cyclin-dependent kinase inhibitors.

Task 1. Characterize a series of breast cancer cell lines which overexpress cyclin D1.

We have begun choosing a set of breast cancer cell lines to analyze for cyclin D1 and p21 expression. As part of this task, we needed a relatively "normal" breast epithelial cell line for comparison. We have chosen the MCF10A line because it is an immortal cell line that arose spontaneously from benign breast tissue without viral or chemical intervention (some of which can disrupt cell cycle regulation). Although MCF10A cells obtained originally from the American Type Tissue Collection (ATCC) were already transformed, we have more recently obtained normal MCF10A cells. In our hands the cells exhibit a requirement for mitogens and adhesion for proliferation. Their doubling time is approximately 24 to 28 hrs. Asynchronous proliferating MCF10A cells exhibit low to moderate but easily detectable levels of cyclin D1. The levels of p21 are undetectable. Furthermore, we have determined that serum starvation for 3 days synchronizes the cells in G0 (Fig 2). We will subsequently begin comparisons

with malignant breast cancer cell lines with regard to cyclin D1 and p21 expression levels.

Aim 4: Examine the relative expression of cyclin D1 and cyclin dependent kinase inhibitors in a series of breast cancer biopsis.

Task 1. Develop and quantify the immunohistochemical procedures for the analysis of breast cancer bipsies. Confirm that the antibodies can detect protein expression in formalin fixed, paraffin-embedded tissue.

We described the procedure for staining cell pellet sections for cyclin D1 and p27 in the last report. We are currently determining the optimal conditions for p21 low and high expression levels and have prepared cell pellets to analyze optimal conditions for p21 staining (see task 2).

Task 2: Obtain high- and low-expression controls for cyclin D1, p21, and p27 antibodies by preparing blocks from cell lines which over- or under-express the cognate protein.

We described the preparation and staining of the positive and negative controls for cyclin D1 and p27 in the last report. Since the p21 antibody is species-specific, mouse fibroblasts cannot be used to obtain the controls for optimization conditions. Using breast epithelial cells, we have determined that cells starved for 2 days completely suppress p21 expression. Conversely, synchronized cells that are stimulated for 2 to 4 hours, maximally express p21 (Fig 3). Using these conditions, we have prepared cell pellets for high- and low-expression controls for p21.

Task 3. Identify human breast cancer tissue specimens which fulfill the requirements for good and poor prognostic factors. Prepare tissue sections of these specimens in a blinded fashion for evaluation.

Dr. Mies has chosen 22 specimens of human breast cancer tissue from the tissue bank at the University of Miami. These include both good and bad prognostic samples but are blinded until the completion of the study.

Task 4. Perform the immunohistochemical staining of the selected specimens using the characterized antibodies. Analyze and quantify the relative expression levels of cyclin D1, p21 and p27 in each biopsy.

Dr. Mies has performed the immunohistochemical staining for cyclin D1 and p27 on the specimens chosen in Task 3. Staining for cyclin D1 has proven to be somewhat difficult because high levels of background staining have made interpretation difficult. After repeating with various reagents, she has obtained more optimal and reproducible staining results. Dr. Mies has also stained the sections for MIB-1/Ki67, an S-phase marker. The results for the

immunohistochemical stains thus far are shown in Table 1 and a representative specimen demonstrating these stains is shown in Fig 4. Once the optimization procedures for p21 staining are determined, these sections will be also be analyzed for p21 expression levels.

Following complete analysis of the results, the clinical correlation and prognostic factors will be unblinded to determine if there is a correlation between expression levels and prognosis.

KEY RESEARCH ACCOMPLISHMENTS

-A tetracycline-regulated cyclin D1 construct has been successfully cloned into mouse embryo fibroblasts and is stable in cell culture. Cyclin D1 expression is normally regulated by adhesion in two of these clones and appropriately regulated by tetracycline in all.

-Utilizing these clones, extensive characterization of the effect of cyclin D1 on p21 expression has been performed. It appears that in mouse embryo fibroblasts, there is no compensatory increase in p21 levels despite significant overexpression of cyclin D1.

-We have characterized the conditions for regulation of cyclin D1, p27 and p21 in MCF10A cells. The characterization of expression levels of cyclin D1 and p21 in breast cancer cells is underway.

-Optimization of immunohistochemical staining procedures using cyclin D1 and p27 antibodies has been completed. Optimization for p21 antibodies is underway.

-Two groups of human breast cancer specimens which exhibit good and poor prognostic factors, respectively, has been selected and stained in a blinded fashion for cyclin D1 and p27.

REPORTABLE OUTCOMES

We have developed MEF cells that are stably transfected with a tetracyline-regulated cyclin D1 construct that are adhesion-dependent and appropriately responsive to tetracycline.

CONCLUSIONS ("so what section")

1. Mouse embryo fibroblasts that stably express a tetracycline-regulated cyclin D1 construct have been successfully established. This system was chosen because p21 and p27 knock-outs were generated in MEFs. Despite

significant overexpression of cyclin D1 in these cells, there was no compensatory upregulation of p21 in MEFs. This is in contrast to the ability of NIH-3T3 cells to upregulate p21 in response to cyclin D1 overexpression. This result precludes the ability to analyze the effect of such interaction on tumor formation of these cells in nude mice. Possible explanations include the acquisition of p53 mutations in MEFs which may interfere with their ability to upregulate p21 as compared to cells with wild-type p53. We plan to use NIH-3T3 cells to determine if the induction of p21 is due to changes in its mRNA expression.

2. A series of human breast cancer specimens have been analyzed for cyclin D1, p27, and MIB-1/Ki67 levels. Analysis of p21 levels will follow completion of optimization of the staining procedure. Following complete analysis of the results, the clinical correlation and prognostic factors will be unblinded to determine if there is a correlation between expression levels and prognosis.

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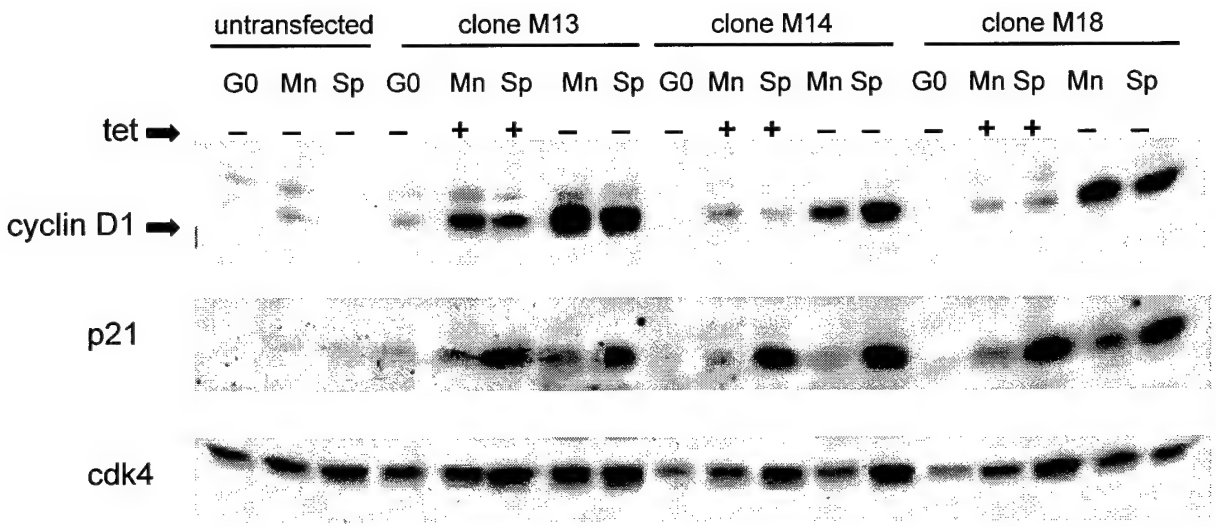
FIGURES

Figure 1. Control (untransfected) MEFs and MEFs stably expressing tetracycline-regulated cyclin D1 were serum-starved into G0, trypsinized, suspended in DMEM, 5% FCS and replated in monolayer (Mn) or suspension (Sp) in the presence (+) and absence (-) of tetracycline for 18 hr. Cells were collected and lysed. Equal amounts of protein from each cell lysate was fractionated on an SDS gel, transferred to nitrocelluolose and immunoblotted with antibodies specific for cyclin D1, p21 and cdk4 (protein loading control).

Figure 2. Synchronization of MCF10A cells by serum and growth factor deprivation Subconfluent cells were seeded in replete media the day prior to starvation to reach 70-80% confluency on Day 0. Cells were washed and the media replaced with DME/F12 without growth factors and serum. Cells were harvested after the indicated number of days and lysates separated by SDS-PAGE. The levels of cyclin D1, cyclin A, p27, and cdk4 (as a loading control) were determined.

Figure 3. p21 induction in MCF10A cells. Cells were synchronized in G0 by serum and growth factor deprivation followed by stimulation with 10% FCS and EGF. Cells were collected at the indicated time points and lysates separated by SDS-PAGE. The levels of p21 and actin (as a loading control) were determined by specific antibodies.

Figure 4. Example of an invasive lobular carcinoma immunohistochemically stained for cyclin D1, p21 and MIB-1/Ki67. a) Cyclin D1 stains the nuclei of the majority of infiltating carcinoma cells dark brown-black; a central benign terminal duct-lobular unit is negative. b) The majority of nuclei are positive for p27. c) anti-MIB-1/Ki67 darkly stains a small number of nuclei in the same carcinoma. (Fast green counterstain; original magnification 200x.)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clone M13
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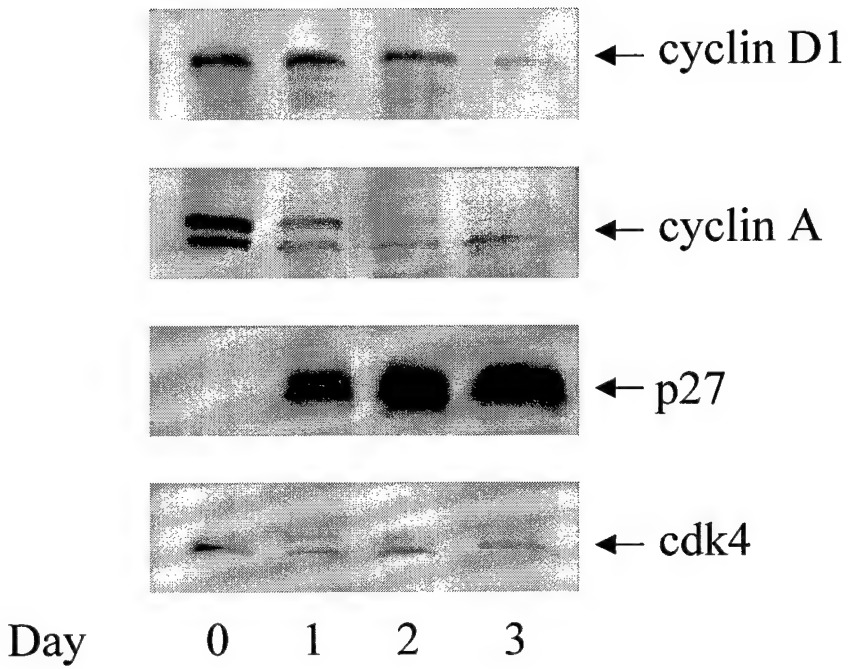
cyclin D1
p21
cdk4

Figure 1



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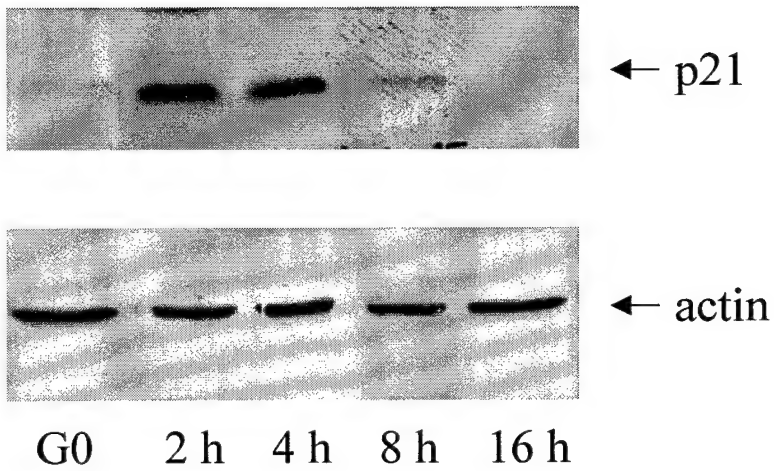

Figure 3

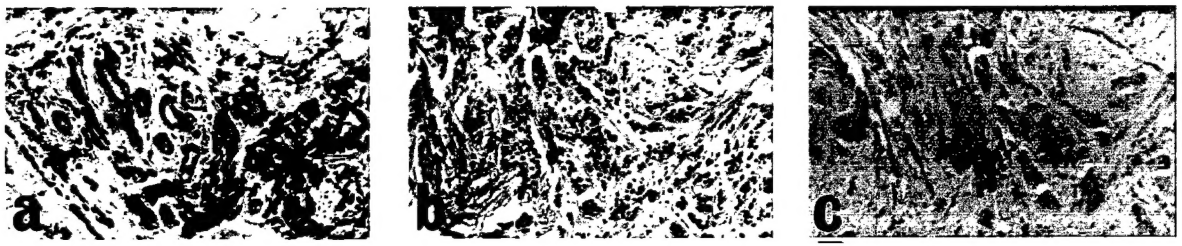

Figure 4

CYCLIN D1, P27 & Ki67 IN INVASIVE LOBULAR CARCINOMA

CASE #	SURGICAL PATH #	MORPHOLOGICAL SUBTYPE	CYCLIN D 1	P27	PENDING P 21	MIB-Y	KI 67
		C= Classical V= Variant P= Pleomorphic					
1		C					
	Invasive	Present	1 +	3 +		1 +	
	In Situ	DCIS Present	1 +	3 +		1 +	
		LCIS Absent	--	--		DCIS	> ILC
2		C					
	Invasive	Present	1 +	3 +		0	
	In Situ	Part of one TDLU	0	NA		NA	
3		C					
		P					
	Invasive	Present	1 +	2 +		1 +	
	In Situ	LCIS Present	2 +	3 +		1 +	
4		P					
	Invasive	Present	2 +	2 +		0	
	In Situ	Present	0	2 +		0	
5		C					
	Invasive	Present	TI	3 +		1 +	
	In Situ	Present	TI	3 +		0	
6		V= Tubulobular					
	Invasive	Present	0	0		Low 1 +	
	In Situ	DCIS Present	0	1 +		1 +	
		LCIS Absent	--	--			
7		P					
	Invasive	Present	2 +	2 +*		1 +	
	In Situ	Present	1 +	3 +		0	

There is a large cell component in the invasive lobular component in which p27 is (-) or very low. Strong (+) staining is seen only in the smaller cells.

Definitions: TI = Technically Inadequate; repeate
Staining Score: 1+ =1-33% cells positive
2+ = 34-66% cells positive
3+ = 67-100% cells positive
0 = Negative
NA = Not aapparent on the section

CYCLIN D1, P27 & Ki67 IN INVASIVE LOBULAR CARCINOMA

CASE #	SURGICAL PATH #	MORPHOLOGICAL SUBTYPE	CYCLIN D 1	P27	PENDING P 21	MIB-Y	KI 67
		C= Classical V= Variant P= Pleomorphic					
8		C					
	Invasive	Present	3 +	1 +		2 +	
	In Situ	Absent	--	--		--	
9		C					
	Invasive	Present	1 +	1 +		2 +	
	In Situ	DCIS Present	1 +	1 +		1 +	
		LCIS Present	1 +	1 +		NA	
10		C					
	Invasive	Present	TI	1 +		1 +	
	In Situ	Absent	TI	--		--	
11		P					
	Invasive	Present	3 +	1 +		1 +	
	In Situ	Absent	--				
12		C					
	Invasive	Present	3+	3 +		2 +	
	In Situ	Absent	--	--		--	
13		P					
	Invasive	Present	1 +	3 +		1 +	
	In Situ	Absent	--	--		--	
14		C					
	Invasive	Present	3 +	rare +		TI	
	In Situ	Present	0				
15		C					
	Invasive	Present	3 +	1 +		1 +	
	In Situ	DCIS Present	3+	1 +		1 +	

Definitions: TI = Technically Inadequate; repeate
Staining Score: 1+ =1-33% cells positive
2+ = 34-66% cells positive
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CYCLIN D1, P27 & Ki67 IN INVASIVE LOBULAR CARCINOMA

CASE #	SURGICAL PATH #	MORPHOLOGICAL SUBTYPE	CYCLIN D 1	P27	PENDING P 21	MIB-Y	KI 67
		C= Classical V= Variant P= Pleomorphic					
16		C					
	Invasive	Present	TI	3+			TI
	In Situ	Present	TI	TI			TI
17		V					
	Invasive	Present	1 +	1 +		1 +	
	In Situ	Absent					
18		C					
	Invasive	Present	3 +	1 +		1 +	
	In Situ	Absent	NA	NA		NA	
19		C					
	Invasive	Present	3 +	1 +		1 +	
	In Situ	Absent	--	--		--	
20		C					
	Invasive	Present	2 +	2 +		0	
	In Situ	DCIS Present	1 +	2 +		1 +	
		LCIS Present	0	2 +			
21		C					
	Invasive	Present	3 +	3 +		1 +	
	In Situ	Present	3 +				
22		C					
	Invasive	Present	1 +	1 +		2 +	
	In Situ	Absent	--	1 +		NA	

Definitions: TI = Technically Inadequate; repeate
Staining Score: 1+ =1-33% cells positive
2+ = 34-66% cells positive
3+ = 67-100% cells positive
0 = Negative
NA = Not aapparent on the section